The final publication is available at Springer via http://dx.doi.org/10.1007/s11160-015-9390-1

Title Misidentification of bluefin tuna larvae: A call for caution and taxonomic reform.

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Abstract: The international effort to prevent the collapse of Atlantic bluefin tuna (BFT, *Thunnus thynnus*, Scombridae) stocks exemplifies the challenges associated with modern marine resource conservation. Rampant mismanagement, under-reporting and Illegal, Unreported and Unregulated fishing led to decades of over-exploitation in the BFT fishery. Surveys of larval abundance in the Gulf of Mexico and the Mediterranean Sea have been used as a proxy for both spawning biomass and recruitment by researchers working to improve estimates of stock abundance. Recent genetic barcoding studies have revealed that species identification errors are common among larvae surveys that use morphology-based taxonomy alone. Misidentification of larvae can lead to uncertainty about the spatial distribution of a species, confusion over life history traits and population dynamics, and potentially disguise the collapse or recovery of localized spawning sites. In an effort to identify the source of these errors, we review several weaknesses in modern morphology-based taxonomy including demographic decline of expert taxonomists, flawed identification keys, reluctance of the taxonomic community to embrace advances in digital communications and a general scarcity of modern user-friendly materials. Recent advances in molecular techniques useful for specimen identification and population studies are discussed at length. We advocate a more constructive integration of morphology-based taxonomy and barcoding in order to add confidence to larval surveys and to strengthen associated fisheries management.

Keywords

Bluefin tuna, fish larvae, barcoding, misidentification, taxonomy, fisheries

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Introduction

There isn't a fish in the sea that better represents the challenges surrounding contemporary marine resource conservation than the Atlantic bluefin tuna (BFT, *Thunnus thynnus,* Scombridae). A bloated EU purse seine fleet, under-reporting by fishermen, increased commercial interest in Japan and sea ranching has resulted in intense over-exploitation of the species. In the last 50 years, the abundance of BFT in the ocean has reduced by more than 70% and their geographic range has contracted by 53% (Worm and Tittensor 2011). In 2006, ICCAT introduced a multi-annual recovery plan (ICCAT 2006) which has contributed to a decreased overall harvest of both adult and juvenile fish and stocks are finally showing signs of improvement (García et al. 2013b; ICCAT 2014a). Although, the rate and nature of this recovery is still very much uncertain, as ICCAT's Standing Committee on Research and Statistics admits, the quality of data currently provided by fishery dependent sources are inadequate to formulate an accurate stock assessment (ICCAT 2013; Fromentin et al. 2014).

Once properly identified, fish eggs and larvae can provide vital information for troubled fisheries, like that of BFT, concerning population structuring, spawning seasons and locations, as well as spawning stock biomass (SSB) or recruitment trends. Significant relationships between SSB and the abundance of eggs and larvae have been observed in several species including small pelagics (anchovy, Pacific and Atlantic mackerel; Lockwood et al. 1981; Lasker 1985; Stratoudakis et al. 2006; Lo et al. 2010) and demersal species (rockfish, cod, plaice, sole; Moser et al. 2000; Armstrong et al. 2001). As such, egg and larval abundance indices are commonly used to calibrate, improve and validate stock assessment models, which are often subject to inaccurate fisheries data (Hsieh et al. 2005). For decades, scientists from ICCAT member nations have been using BFT larval indices generated from surveys conducted in the Gulf of Mexico to calibrate Virtual Population Analyses for the western stock (Scott et al. 1993; Scott and Turner 2003; Ingram et al. 2010). In 2013, the first standardized BFT larval indices for a Mediterranean spawning site were published based on larval surveys conducted by the Spanish Institute of Oceanography around the Balearic Islands in the western Mediterranean (Ingram et al. 2013). Temporal shifts in BFT larvae abundance and condition can provide important information about recruitment success relative to short and long term environmental changes (Alemany et al. 2010; Lindo-Atichati et al. 2012; García et al. 2013a). Larval surveys can also help to determine community assemblage dynamics in order to develop additional data for the development of ecosystem-based management approaches (Richardson et al. 2010). Surveys of this nature have revealed that highly exploited species exhibit more variability in abundance in response to climate change than under-utilized species (Hsieh et al. 2006). In the context of a rapidly changing environment and a swollen fishing fleet, our ability to accurately identify and monitor BFT throughout its life history is critical for their effective management.

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Clearly, the distribution and quantity of early life stage fishes can provide a wealth of information but what happens when eggs and larvae are classified and counted incorrectly? Surveys monitoring the abundance of fish eggs collected in the Irish Sea in 2001 were used by ICES to estimate the SSB of cod (*Gadus morhua*, Gadidae), plaice (*Pleuronectes platessa*, Pleuronectidae) and sole (*Solea solea*, Soleidae). The resulting estimates far exceeded those given by Virtual Population Analysis (Armstrong et al. 2001). Subsequent genetic analysis revealed that many of the eggs had been misidentified, leading to large over-estimations of cod SSB (Fox et al. 2005). Elsewhere, inaccurate estimates of egg diameters used to identify sciaenids (Sciaenidae, Perciformes) may have led to over-estimations of up to 50% of the SSB of black drums, *Pogonias cromis,* in Chesapeake Bay (Daniel and Graves 1994). A recent study focusing on the ability of researchers from five different laboratories in Taiwan to identify larvae determined that the average accuracy of identification was 80.1%, 41.1% and 13.5% at family, genus and species levels, respectively (Ko et al. 2013). Families containing the most misidentified larvae in that study were Sparidae, Scorpaenidae, Scombridae, Serranidae and Malacanthidae. Recently, Puncher et al. (submitted – note to editor) revealed that Atlantic bluefin tuna have been misidentified in the Mediterranean Sea. In that study, they showed that more than half of larvae submitted by three Mediterranean institutions to an ICCAT funded BFT research project were the wrong species.

In this review, we explore the events leading up to the misidentification of BFT larvae in the Mediterranean and present the argument that additional mistakes are likely to have occurred in the past and have a high likelihood of happening again in the future unless corrective measures are taken. In order to establish context for our concerns, we first provide a small review of problematic assumptions that have been made based upon what we have learned from young fish that have been identified as BFT. This is followed by a review of the problems associated with morphology-based taxonomy and its efficacy as a primary tool within the framework of large-scale tuna larvae surveys. We critically examine the resources available to survey teams and make several suggestions for improvements. Finally, we review the merits of morphology-based taxonomy and molecular techniques and highlight the need for a harmonization of both methods in order to minimize costs and optimize wildlife conservation and fishery management efforts.

A Context for Concern

Larval surveys have confirmed that BFT spawn during May and June in the eastern Mediterranean, June and July in the western Mediterranean (Duclerc et al. 1973; Alemany et al. 2006; Heinisch et al. 2008), and April to June in the Gulf of Mexico and Florida Straits (Baglin 1976; Richards 1976; Fig. 1). BFT larvae have also been observed in the Gulf of Guinea during February, March and August (Richards 1976), as well as the Black Sea during summer months, until they disappeared from that body of water in the 1980s (Vodianitskii and Kazanova 1954; Akyuz and Artuz 1957). Significant numbers of BFT larvae have also been identified off the coasts of Turkey (Bay of Mersin) and Tunisia (Oray and Karakulak 2005; Giovanardi and Romanelli 2010; Zarrad et al. 2013; Koched et al. 2013). In 1983, Piccinetti and

Piccinetti Manfrin (1993) identified BFT larvae off the northern coast of Egypt; however, their presence in those waters has not been verified since. Some researchers have suggested that spawning may also occur in the mid-Atlantic, after tagged adults were found there during spawning months (Lutcavage et al. 1999). Of course, this might also be evidence that BFT do not necessarily spawn annually (Galuardi et al. 2010). The much restricted BFT fishing season in most parts of the Mediterranean Sea is scheduled to take place during these spawning periods (currently 26 May to 24 June; ICCAT 2014b). Many other fisheries choose to safeguard mature fish from capture until spawning has completed (Grüss et al. 2014). Of course, many (not all) of the BFT captured in the Mediterranean Sea are there for a few weeks alone, for the sole purpose of reproduction (Aranda et al. 2013) and a great deal of revenue would be lost if this season were to be closed. Future BFT conservation efforts may focus on protection of spawning areas and seasons, as they have been previously identified by the presence of their eggs and larvae.

Much of our understanding concerning favourable environmental conditions for spawning of BFT comes from research based in the western Mediterranean Sea. Larval surveys in this area have shown that changes in relative abundances of different species are directly influenced by hydrodynamics (Alemany et al. 2010; Reglero et al. 2012). BFT larvae are most abundant in surface waters ranging between 23-28°C, where two water masses collide and create complex hydrodynamic conditions lacking strong, directional flows (García et al. 2005a; Reglero et al. 2012; Muhling et al. 2013). As Mediterranean waters are generally oligotrophic and do not provide ample food for the high bio-energetic demands of quickly developing BFT larvae, spawning typically occurs at the formation of frontal structures, the boundaries of anti-cyclonic gyres, and upwelling zones typical of oceanic islands, wherein both food particles and larvae are entrained (García et al. 2005a; Aguilar et al. 2009; Mariani et al. 2010; Lindo-Atichati et al. 2012). The Balearic Islands in the western Mediterranean are arguably the most productive of BFT spawning areas (Alemany 2008; García et al. 2013b). Within these waters, BFT larvae tend to be more abundant in areas where incoming Atlantic water masses and resident surface Atlantic waters, already modified by a longer stay in the Mediterranean, converge (García et al. 2005a; Alemany et al. 2006, 2010; Reglero et al. 2012). The discovery of low nutrient concentrations and reduced primary production in some areas with high concentrations of larvae have led some researchers to suggest that predation on non-BFT fish larvae and cannibalism plays an important role in the survival and development of BFT recruits. The continual spawning of BFT seen over a period of weeks produces sub-cohort overlap, exposing smaller larvae to predation by older recruits; a behaviour that appears to be favoured by higher sea temperatures, when growth rates increase and size classes become more distinct (Reglero et al. 2011; García et al. 2013a). The Tyrrhenian and Ionian Seas also produce high quantities of BFT larvae, particularly off the south eastern coast of Sicily (Cape Passero), where the confluence of the Atlantic Ionian Stream and upwellings result in a retention area rich in potential prey items (Lafuente et al. 2002; García et al. 2005b). Predictions concerning the impacts that climate change and future ocean

conditions will have on BFT populations will be based on these larval abundance studies. Unfortunately, this research is taking place on a limited geographic scale and until additional resources (monetary and human expertise) are allocated elsewhere, this will continue to be the case.

Our current understanding of BFT spawning in the Mediterranean Sea suggests that the western basin is the most productive source of young bluefin tuna; however, spawning also takes place in the central and eastern Mediterranean Sea. Discrepancies in larval survey results from this region have cast some doubt on the location, consistency, timing and success of spawning events. For example, a 1994 larval survey of the entire Mediterranean collected 1160 tuna larvae, including 183 BFT, none of which were caught in the Levantine Sea (Piccinetti *et al.* 1997). These results are not surprisingly, since the survey was conducted between 21 June and 7 July, several weeks after the established spawning season (Karakulak *et al.* 2004) and south of Cyprus, instead of in the Bays of Antalya and Mersin, north of the island, where BFT larvae are often found (Oray and Karakulak 2005). Japanese surveys have reported low densities of BFT larvae in hauls taken in the mid-Levantine Sea around the Herodotus Basin (Tsuji *et al.* 1997, Nishida *et al.* 1998). These larvae may have originated at the periphery of the Rhodes and Léraptra anti-cyclonic gyres, which are composed of inflowing Atlantic waters south of the islands of Rhodes and Crete, respectively; a hypothesis that fulfils both the hydrodynamic and island proximity requirements for BFT spawning (Robinson *et al.* 1992). Waters exiting these gyres to the south are carried eastward by the Mid-Mediterranean Jet Stream and later bifurcate south to the waters of the Herodotus Basin and north to the Bay of Antalya. Alternatively, spawning may also be occurring between the island of Cyprus and the coastlines of Syria and Lebanon, where a number of smaller seasonal anti-cyclonic gyres form. These water masses are carried northward by the Cilician Current along the southern coast of Asia Minor, where they can be retained in eddies formed in the Bays of Mersin and Antalya (Robinson *et al.* 1992, Özsoy *et al.* 1993).

BFT larvae are commonly transported over significant distances by both jet stream waters and meandering gyres throughout their distribution. Larvae appear to accumulate in retention areas with high concentrations of food particles (including conspecific larvae), wherein they develop into juveniles and migrate en masse to nursery areas (McGowan and Richards 1989). In the Western Atlantic, seven-day-old BFT larvae (>4.0 mm) have been captured off the coast of North Carolina using a mesh size of 0.33m, well outside of known spawning areas (McGowan and Richards 1989). Although ocean currents in that region are capable of transporting larvae from the Gulf of Mexico and Straits of Florida, approximately 970 km, the origin of larvae around Cape Hatteras is unkown. The majority of BFT larvae captured in that area have been large (4.6 - 6.5 mm), while smaller larvae (<3.5 mm) are noticeablty absent among samples. Similarly, the majority (65%) of larvae captured in the northern Levantine Sea have been large (5-7 mm, approximately 10 days post hatch), opening up the possibility that their origin may lie in a different body of water;

although, some smaller larvae (3.1 mm) have also been captured (Oray and Karakulak 2005). Moreover, most larval surveys conducted in the Levantine Sea have used nets with a 1 mm mesh size which are unlikely to retain the entire smaller size fraction of larvae (Oray and Karakulak 2005). Therefore, it is currently inadvisable to compare the results of larval surveys taking place in the eastern Mediterranean with other locations, since they cannot be considered as quantitative hauls. Clearly, the execution of additional standardized larval surveys, supported with genetic identification of samples and hydrographic modelling, are required in the Levantine Sea to the extent that they have been performed elsewhere in the Mediterranean Sea.

Potential for errors in BFT larvae identification

In general, identification of tuna eggs and the larvae of some tuna species using morphological characteristics alone is incredibly difficult, requiring an in depth knowledge of taxonomy as well as patience and experience. Two of the world's former leading tuna taxonomists warned that "the young stages of fishes of the family Scombridae are among the most difficult to identify to generic levels and particularly to the species level" (Richards and Pothoff 1974). Kohno et al. (1982) cautioned that an "extensive knowledge of individual, growth-associated, and geographic variations" in patterns of melanophores is required to make an accurate identification of *Thunnus* species. Unique patterns of red pigmentation have also been used to distinguish species; however, this type of pigmentation is most prominent in larvae caught during the night (Matsumoto et al. 1972) and is lost during formalin fixation and ethanol preservation (Richards et al. 1990). Due to the difficulties associated with correctly identifying larger larvae (>5 mm) by pigmentation patterns alone, some authors advise using osteological characteristics; however the process of clearing, staining and examining is time consuming, unfeasible for large surveys and often results in additional loss of samples (Richards et al. 1990). Identification based upon morphometrics alone is unfeasible due to body distortions occurring during fixation or rapid growth spurts that distend and stretch the body during rapid early development (Matsumoto et al. 1972). Making matters worse, fish larvae are often so damaged during sampling that identification to species level using morphological features becomes impossible (Paine et al. 2007). Due to the scarcity of expertise in larval taxonomy, species identification is commonly outsourced to distant laboratories that specialize in the sorting of plankton (Scott et al. 1993; Ingram et al. 2010; Matarese et al. 2011; Lindo-Atichati et al. 2012). Some researchers have resorted to limiting identification of their captured larvae to lower taxonomic levels (Hernandez et al. 2010; Lindo-Atichati et al. 2012), while others have turned to genetics to identify to species level (Chow et al. 2003, 2006).

The ability of field technicians to identify larvae to species level is limited by the quality of materials and tools that they are given to accomplish this challenging task. In some fields of study, misidentification of species can reach as high as 90%, which can lead to serious errors concerning community composition and population inferences (Vecchione et al. 2000). Decades ago, before recent clarification of tuna systematics, Richards (1976), a reputed

ichthyoplanktologist warned, “For a group like the tunas I am sceptical of reports based on eggs and early larvae unless these young stages have been raised to identifiable sizes.” Unfortunately, culturing unknown eggs and larvae until they can be accurately identified has proven extremely difficult for some long-range pelagic species, including BFT (Hyde et al. 2005). Rearing of BFT from eggs through to their juvenile stages has been completed only recently (de la Gandara et al. 2010). As a result, most larval surveys rely on a small handful of taxonomic keys, illustrations and journal publications for species identification (unless one of the world’s few tuna taxonomists or a well-trained technician is onboard to assist with the painstaking task of identifying thousands of larvae). When sourcing texts that specialize in BFT systematics, confusion quickly ensues as most texts are quite old and use several of 15 synonyms, seven of which were generated by Linnaeus alone (Froese and Pauly 2015). A few keys are easily obtained on the internet, while most require a personal contact with someone in the industry willing to share digital copies. Obviously since most texts are several decades old, there is no standardized formatting or consistency in quality. Several texts that are routinely referenced by current larval studies are written in Japanese (Ueyanagi 1966, Yabe 1966), Italian (Sanzo 1932a; Scaccini 1975), French (Duclerc et al. 1973) and Spanish (Dicenta 1975). Some of these keys are very old and after several generations of scanning and copying of illustrations, morphological features contained therein have become difficult to distinguish.

Early taxonomic descriptions of scombrids were often based on misidentified larvae or eggs and are thus riddled with errors and confusion (Alemany 2008). The unreliable nature of these guides was identified as early as 1976 by Duclerc et al. (1976), who called them contradictory and complained that they made use of too many different criteria for comprehensive identifications. The authors of some of these early works have admitted that errors were made when identifying the larvae that were later used to build identification keys (Matsumoto et al. 1972; Richards and Pothoff 1974). Unfortunately, efforts to replace these early works with accurate descriptions have yet to take place. As a result, successive publications based on these works have carried the errors forward. The origin of these mistakes are the pioneer works on tuna larvae in the Mediterranean in which BFT larvae and those of other tuna species were misidentified, namely Ehrenbaum (1924) and Sanzo (1932). For example, the larvae identified by Ehrenbaum (1924) as *T. thynnus* were in all likelihood *T. alalunga*, according to the pigmentation pattern of the specimens illustrated in their publication. In short, the illustrated larvae all but lack diagnostic black pigmentation in the trunk, except for a few dorsal and/or ventral melanophores on the urostile, a pattern that is maintained from hatching to advanced postlarval stages (Alemany 1997 and references therein) and a characteristic that is not shared by any other tuna species inhabiting the Mediterranean Sea. Using fertilized eggs captured at sea, Sanzo (1932,1933) described eggs and early hatched larvae of *T. thynnus* and *T. alalunga* (at that time *Orcynus thynnus* and *Orcynus germo*, respectively), following a comparison with mature eggs extracted from the ovaries of captured adult females. However, the continuous row of

ventral melanophores, preanal ventral pigmentation and few dorsal melanophores in the caudal area featured in the resulting drawings and descriptions suggest that Sanzo (1932,1933) was unintentionally working with *A. rochei* specimens (Alemany 1997 and references therein) and not *T. thynnus* and *T. alalunga* as intended. Later, Scaccini et al. (1973) confirmed these errors when eggs identified as BFT, according to Sanzo's descriptions, released larvae belonging to other species. In fact, larvae identified by Scaccini et al. (1973) as BFT were probably *A. rochei,* based on a photograph featured in the publication of a recently hatched larva. These two erroneous works by Ehrenbaum (1924) and Sanzo (1932) were the only references taken into account by Padoa (1956) for describing BFT larvae, in what has become one of the most extensively used fish larvae identification guides for the Mediterranean Sea. From these dubious origins, drawings and descriptions of early life stage BFT and albacore by Sanzo (1932, 1933) continue to resurface in fish larvae identification guides of species inhabiting in the Atlantic Ocean, including Fritzsche (1978), Fahay (2007) and Richards (2005).

Confusion between illustrations and descriptions of *T. thynnus* and *T. orientalis* has also persisted for decades as many of the most relied upon guides predate the separation of the two species (Collette 1999). For example, Yabe et al. (1966) published an in depth description of *T. orientalis* and *T. maccoyii* and their distribution in the Pacific Ocean, containing eight detailed illustrations of *T. orientalis* larvae. Jones et al. (1978) then used the illustrations by Yabe et al. (1966) as well as several other drawings of *T. orientalis* by Ueyanagi and Watanabe (1964) and Ueyanagi (1966) for their in depth description of *T. thynnus*. Fahay continued this practice of recycling illustrations in two publications in 1983 and 2007 in which they use the same drawings by Yabe et al. (1966). In their 1983 publication, they indicate in a footnote that the *T. thynnus* larvae were "Pacific material"; however, this important detail was omitted from their later publication in 2007 (Fahay 1983, 2007). It is unfortunate that this occurred, considering that this key was published years after the *Thunnus orientalis* species designation in 1999. Certainly, these mistakes in the pioneer works have confounded many scientists for decades and are undoubtedly the root of the issue surrounding BFT misidentifications.

During the 1970s several attempts were made to clarify the confusion surrounding the taxonomy of early life stage scombrids. In a thorough review, Richards and Pothoff (1974) warned that established osteological diagnostic features sometimes contradict conclusions based on pigmentation. Later, Dicenta (1975) noted encountering a great amount of difficulty when attempting to differentiate between the eggs and larvae of *T. thynnus* and *A. thazard* (the authors likely meant *A. rochei*). Following the examples provided by Sanzo (1932, 1933), Scaccini (1975) claimed that there are no differences to be found in the pigmentation of *T. thynnus* and *T. alalunga* at sizes smaller than 5 mm. Soon after Kohno et al. (1982) identified 191 larvae as *T. thynnus* and *T. alalunga* using guides by Scaccini et al. (1975) and Fritzche (1978), among others, and determined that the patterns of melanophore distribution were inconsistent within each species, concluding that the use of pigments as diagnostic features was limited. Since all of these studies were

working from the same erroneous guides, it comes as no surprise that new sources of confusion, such as intraspecific variation (resulting from *A. rochei* specimens analyzed as *T. thynnus*), were discovered and propagated.

It has now been over two decades since this literature-based discussion fell silent and although early life stage scombrid taxonomy has since been resolved, a comprehensive review clarifying the confusion and identifying errors contained in existing identification guides has yet to be published. Modern internet-based standards are disappointing in terms of illustrations used, or lack thereof. FishBase (Froese and Pauly 2015) features an illustration of a 25-day-old juvenile *T. orientalis* incorrectly labelled as *T. thynnus* (Miyashita et al. 2001), while ICCAT's species identification sheets contain a single illustration of a juvenile *T. thynnus*. As Godfray (2002) pointed out, the quantity of "taxonomic information available on the web is pitiful, and what is present (typically simple lists) is of little use to non-taxonomists". Tunas are obviously no exception to this generalization and new efforts should be made to definitively identify and describe each species different larval morphotypes with the support of genetic analyses and/or conclusive rearing experiments.

As a first contribution to this effort, we offer a clarification of some of the more contentious elements affecting the correct identification of BFT larvae in the Mediterranean. Currently, there are no correct and complete descriptions of BFT larval development published, including illustrations of the different developmental stages from eggs and yolk sac larvae to advanced postlarvae, based on Mediterranean material. Despite the scarcity of descriptive materials, several incomplete yet useful references do exist. For example, Alemany (1997) contains an original illustration of Mediterranean BFT flexion larvae and the larval stages following yolk sac reabsorption have already been correctly described by Dicenta (1975), who pointed out that the most distinctive species specific feature of these larvae is the presence of dorsal melanophores located mid-trunk. Despite some differences that may exist between the pigmentation pattern of Pacific and Atlantic BFT (Jones et al. 1978; Kohno et al. 1982), the flexion and postflexion stages of *T. thynnus* can be identified using the descriptions of *T. orientalis* larvae in Ueyanagi and Watanabe (1964), Ueyanagi (1966) and Yabe et al. (1966). Moreover, recent rearing experiments carried out in the Mediterranean (de la Gándara et al. 2010) have corroborated the observations provided by Kaji et al. (1996), confirming that *T. orientalis* and *T. thynnus* yolk sac larvae have the same pigmentation pattern; namely a very large dorsal melanophore in the middle of the trunk, which extends to the primordial fin (Fig. 2a). After the yolk sac has been absorbed, this melanophore becomes smaller and migrates to the trunk, giving rise to additional small dorsal melanophores and the typical dorsal melanophore pattern of BFT larvae (Fig. 2b,c). This unmistakable pigmentation pattern can also be observed in larvae that have been preserved in formalin (Fig. 3). Bullet tuna, *A. rochei*, are also found in the Mediterranean Sea and have similar conspicuous dorsal pigmentation at flexion and preflexion stages; however, this is restricted to a few melanophores in the caudal peduncle area which extend anteriorly as the larvae develop (Alemany 1997). Other characteristics, such as

ventral pigmentation (digestive organs and cleithrum) in *Auxis spp.* and a much more intense black pigmentation of the first dorsal fin in BFT, allow for the discrimination of the two species at similar developmental stages. In summary, it is possible to identify scombrid larvae found in the Mediterranean Sea based on morphological characters alone, unfortunately overcoming the confusion cultivated in the past is an obstacle that will be difficult to overcome until accurate and complete descriptions are published.

Required modernization of larval taxonomy

BFT larval surveying efforts would greatly benefit from a modern revision of outdated taxonomic keys and focus should be given to providing new keys and high quality photographs and illustrations online in a digital format. Over a decade ago, Godfray (2002) suggested that a revitalization of taxonomy is required, advocating that it is ideally suited for Information and Communication Technologies because of its information rich nature and large number of meticulously crafted illustrations. Still, for the most part, taxonomy simply hasn't made the technological leap to modern digital communication tools. One may argue that taxonomy, as a science, is being left behind in an age of information that demands a digital interface for nearly all tasks performed in modern research. Guerra-García et al. (2008) declared that taxonomy is in a crisis, warning that funding for taxonomy is generally inadequate and that expert taxonomists are few and in demographic decline. Over a decade ago, Boero (2001) commented on the state of decline of taxonomy, remarking that since publication records of researchers are used as a performance measure, the dismal if not non-existent impact factors of taxonomic journals are not attractive for young researchers trying to establish themselves in their careers. As such, the number of professional and amateur taxonomists has undergone a long and persistent decline since the 1950s (Hopkins and Freckleton 2002). In 2003, Wilson (2003) estimated that as few as 6,000 biologists were working in taxonomy worldwide. The culmination of these numerous pitfalls has slowed the progress of species identification and the assessment of global biodiversity; an ethereal obstacle now described as the "Taxonomic Impediment". The disadvantages associated with morphological taxonomy weighed against the increasing efficiency of molecular techniques are driving more researchers towards DNA barcoding for routine species identification.

A heated debate has been raging between advocates of morphological and molecular taxonomy since the inception of DNA barcoding. Some opponents of molecular taxonomy have argued that DNA barcoding explores a very restricted portion of the genome and this may lead to systematic errors in classification. They argue that the use of a single standard sequence is equivalent to classifying organisms based upon a single morphological feature (Tautz et al. 2002). Seberg et al. (2003) expressed concerns over a monopolization of information by wealthier nations, warning that an expensive and centralized DNA-based taxonomy would add to an existing North-South divide in taxonomy. It could be argued that the open-access nature of online sequence databases is acting to decentralize knowledge that was once

the sole possession of aged taxonomists with years of education unavailable to many. It is for this reason that some have claimed that "DNA barcoding promises to entirely democratize the taxonomic process" (Packer et al. 2009).

Others have suggested that the merits of morphological taxonomy and barcoding can combine to improve systematics overall (Stevens et al. 2011; White and Last 2012). DNA barcoding has the potential to accelerate species identifications and diversity assessments by increasing the synergy between field biologists, reference collections and sequencing facilities (Swartz et al. 2008). A key directive of centralized DNA barcoding efforts, such as the Barcode of Life Initiative, is matching known species with archived voucher specimens to DNA sequences (Ratnasingham and Hebert 2007). Likewise, morphological taxonomists are needed to recognize and describe new species as well as confront the daunting task of matching pre-existing descriptions with ever-expanding sequence libraries (Tautz et al. 2002). Similar efforts must also focus on the taxonomic description of early life stage fishes. Kendall and Matarese (1994) estimated that only 10% of the described marine fish species had known larvae and as few as 4% had published descriptions of their eggs. Certainly barcoding has a role to play in these efforts.

Advances in molecular analysis

Several genetic techniques have been used by researchers over the past two decades to identify early life stages of fishes and resolve previously unexplored challenges in fisheries science (Daniel and Graves 1994; Vandersea et al. 2008; Boley and Heist 2011). The most commonly used molecular technique for identification of fish species is the PCR amplification and sequencing of a ~650bp fragment of the cytochrome oxidase subunit 1 (*CO1*) gene located in the mitochondrial genome. The *CO1* gene has been proposed as the basis for a global bio-identification system for all animals (or Barcode of Life Project) for the following reasons: 1) variability of sequences between individuals of the same species is negligible when compared to the variation between species, 2) moderate mutation rate, and 3) characteristic flanking regions that require a limited number of "universal" primers (Hebert et al. 2003). An extension of the Barcode of Life project is the Fish Barcode of Life Initiative (FISH-BOL), which seeks to barcode all of the estimated 32,257 recognized species of fish using the same gene (http://www.fishbol.org; Ward et al. 2009). Barcoding by *CO1* sequences has been used to identify the diversity of reef fish larvae in the Pacific Society Islands (Hubert et al. 2010), Great Barrier Reef (Pegg et al. 2006) and Caribbean Sea (Victor et al. 2009; Valdez-Moreno et al. 2010; Baldwin et al. 2011). *CO1* barcodes have also been used to verify the species of difficult to identify larvae of snapper in the Straits of Florida (D'Alessandro et al. 2010), mackerel off of south-eastern Australia (Neira and Keane 2008), sculpin in the Bering Sea (Matarese et al. 2011), scombrids in the western Atlantic and mid-Pacific (Paine et al. 2007, 2008), sandlance in the Yellow, East and Bering Seas (Kim et al. 2010), and entire communities of medium-sized pelagic larvae in the Straits of Florida (Richardson et al. 2010). Other genes neighbouring the *CO1* gene, such as the ATCO region and 16S rRNA gene, have been used to identify *Thunnus* eggs and larvae offshore from the Mariana Islands and

Yucatán Peninsula, respectively (Kawakami et al. 2010; Muhling et al. 2011); however, molecular barcoding techniques have yet to be applied to large-scale larval surveys focusing on *Thunnus thynnus*. The application of these techniques has provided many solutions to long unanswered mysteries; however, with every advance new challenges are inevitably encountered.

As with many other aspects of working with young tunas, identification of species by *CO1* sequences is not a straightforward enterprise. Due to the similarity between species in the *Thunnus* genus, some researchers have experienced difficulty when identifying unknown samples using genetic distance based approaches, like phylogenetic tree construction (Alvarado Bremer et al. 1997; Paine et al. 2007). Viñas and Tudela (2009) were unable to resolve *Thunnus orientalis* and *T. thynnus* clades using a handful of sequences (n=4-8) and as a result advocated for the use of the mitochondrial control region as an alternative marker for the barcoding of tunas. Since the geographic distribution of *T. orientalis* does not overlap with the spawning areas of *T. thynnus*, the *CO1* marker can be used as a barcoding marker for tuna larvae collected in the Gulf of Mexico and Mediterranean Sea. By using a different analytical approach, Lowenstein et al. (2009) identified seven nucleotides in the *CO1* sequence that distinguish *T. thynnus* for *T. orientalis* and one simple pure characteristic attribute that differentiates *T. thynnus* from all other tunas. As such, the use of individual diagnostic loci to identify species, or characteristic attribute keys, has proven effective for differentiation of scombrid species (Lowenstein et al. 2009). Barcoding of BFT mitochondrial DNA has also revealed that 2-3.3% of Atlantic BFT share nearly identical mtDNA with albacore and Pacific bluefin tunas, due to past hybridization events and subsequent introgression of the mitochondrial genome (Viñas et al. 2011; Alvarado Bremer et al. 2005). As such, nuclear markers, like the rDNA first internal transcribed spacer (*ITS1*) region, has been advocated by some as a means by which albacore and BFT can be differentiated (Chow and Kishino 1995; Chow et al. 2006; Paine et al. 2007; Viñas and Tudela 2009). Using the sequencing approach and tools described above, identification of unknown scombrid larvae in the Mediterranean Sea is straightforward and unambiguous.

Once a species' DNA barcode is published, researchers are able to develop a suite of genetic tools capable of identifying that species without direct sequencing of DNA. A 33-probe suspension bead array has been used to identify 23 different species of California marine fish eggs, including commercially relevant species such as Pacific mackerel (*S. japonicus*), hake (*Merluccius productus*, Merlucciidae), Pacific barracuda (*Sphyraena argentea*, Sphyraenidae), white seabass (*Atractoscion nobilis*, Sciaenidae), California halibut (*Paralichthys californicus*, Paralichthyidae), and diamond turbot (*Hypsopsetta guttulata*, Pleuronectidae) (Gleason and Burton 2012). The developers of this method have suggested that their DNA probes could be scaled up to simultaneously identify the eggs of as many as 100 species. More traditional and economic techniques such as multiplexing of haplotype-specific PCR products and electrophoresis have been used to rapidly identify collections of clupeiform larvae captured in Senegal (Durand et al. 2010). Similarly,

researchers in Hawaii have developed a species-specific set of primers that, when combined with electrophoresis, were capable of distinguishing the larvae of six species of billfish, two species of dolphinfish and wahoo (*Acanthocybium solandri,* Scombridae) onboard a research vessel in only 3 hours (Hyde et al. 2005). Advances have also been made to simplify the process of distinguishing scombrid larvae. For example, Paine et al. (2008) developed a quick molecular diagnostic tool capable of differentiating albacore and bluefin tuna using a restriction enzyme (*Eagl*) to digest amplified fragments of *ITS1*.

Next Generation Sequencing (NGS) is a rapidly evolving array of technologies now being widely employed for fisheries management tasks. SNP panels generated through NGS have been developed in order to discriminate species and populations of albacore tuna, Pacific lamprey and blue catfish (Albaina et al. 2013; Hess et al. 2014; Li et al. 2014). In Europe and America, SNP panels have been used to distinguish between species and populations of salmon as well as farm raised and wild fish (Amish et al. 2012; Drywa et al. 2014; Houston et al. 2014; Larson et al. 2014). The expectation is that these panels will be used for hatchery and marketplace traceability, improvement of breeding programs and wildlife conservation efforts. For example, ICCAT, through the Atlantic-wide Research Programme for Bluefin Tuna (GBYP), is currently funding research to develop a high performance array capable of distinguishing populations of bluefin tuna. NGS technologies also provide the opportunity to quickly ascertain the species composition of entire communities of plankton. Researchers at the Plymouth Marine Laboratory in England recently compared the results of a plankton survey in which species were identified via both a metagenetic and morphological approach (Lindeque et al. 2013). Using 454 pyrosequencing they identified 135 operational taxonomic units (OTUs), over twice the number (58) identified using a morphological approach, including rare species and parasites. This approach may not be capable of giving an exact number of larvae by species collected in a given sample but it can give a relative abundance of each. Fisheries organizations and multi-institutional larval surveys, plagued by sampling and species identification bias, have much to gain from this new technology. Although NGS techniques have been very expensive in the past, its cost is declining rapidly and new markets are opening up in the developing world (Willette et al. 2014).

NGS can produce millions of barcodes per day, the cost of which is decreasing at a much higher rate than predicted by Moore's Law, according to the statistics kept by the National Human Genome Research Institute. Stein et al. (2014) conducted an in depth analysis of the costs associated with the sorting and identification of freshwater macroinvertebrates, fish and diatoms using morphology-based taxonomy, sequencing and the metagenetic analysis of bulk samples of organisms using the IonTorrent PGM™ platform. The cost and time estimates required to obtain results using these separate approaches were based on data provided by commercial laboratories in the USA between 2010 and 2012. Their estimates did not include the cost of sample collection (common for all approaches) and are comparable to what would be paid by a large fishery-based larval survey. They concluded that Sanger sequencing costs 1.7 - 3.4 times

more than a morphology-based taxonomic approach (1200-1700USD per 1000 fish). Although the bulk sample NGS approach is incapable of identifying individual fish and provides relative abundances of each taxa instead, its cost were comparable or slightly less than the morphology-based approach. The time and costs required for Sanger sequencing includes the same steps as traditional methods (sorting of samples, removal of debris, voucher specimen analysis) with additional DNA extraction, PCR amplification and sequencing, adding another 5 USD in cost per individual analyzed. Despite these additional steps, the Sanger sequencing approach is able to provide results within days or weeks of sampling (depending on access to sequencing services), compared to the months required for traditional morphology-based identifications. Modern NGS platforms can provide results in 3 - 5 days, as it eliminates the time required for sorting of the specimens, and costs between 0.50 – 2 USD per individual. However, Stein et al. (2014) believe that Sanger sequencing is more accurate than NGS analysis due to shorter sequence lengths and amplification biases. Researchers are now beginning to use aspects of each approach that optimize their resource costs: use of voucher specimens identified by a morphology-based approach, NGS techniques to discover new barcoding sites or diagnostic/informative loci, followed by Sanger sequencing of low numbers of samples or genotyping of high numbers of individuals with microarrays (Shokralla et al. 2014).

Guide of good practice in BFT larval studies

Due to the unreliable, inconsistent and incomplete nature of catch data provided to tuna fishery scientists, managers are increasingly looking to alternative sources of information. If larval and egg abundances are to be more widely used by tuna fishery scientists, it is imperative that problems associated with sample identification are resolved. Considering that tuna taxonomy has suffered from confusion in the past and is in need of modernization it comes as no surprise that an understanding of BFT larval dynamics remains incomplete. We recommend an elaboration of high quality drawings of the complete developmental series of BFT, accompanied by descriptions based on a high number of individuals from throughout the species range in order to account for intraspecies variability. High definition photographs should be paired with each drawing to help technicians recognize diagnostic features in more realistic and familiar representations of each larval stage. Equipped with these new tools, efforts should be made to confirm that spawning is taking place in areas where BFT larvae have been documented only once or have been identified by only one group of researchers or technicians. If natal homing is indeed a reproductive strategy of BFT, conservation efforts should target remote locations where only a few larvae have been captured, so that rare genotypes can be catalogued. BFT originating in these areas are likely to possess specialized adaptations (egg buoyancy, sperm motility and larval growth rates) to unique local environmental conditions (temperature, salinity, density, current velocity, nutrient availability etc.) (Mackenzie and Mariani 2012), which may prove important for the species' survival in future oceans modified by climate change (Hobday et al. 2015). Regardless of the sampling location, nets with 0.33-0.50 mm mesh

size should be employed with a tow velocity of two nautical kilometres per hour in order to ensure collection of smaller size fractions of larvae. This would allow a comparison of yields across all geographic areas.

Advances in NGS and historical DNA analysis are allowing researchers new access to archived samples (larvae, scales, otoliths, spines and vertebrae), revealing important ecological and evolutionary changes in fish stocks and populations over time (Riccioni et al. 2010; Cuveliers et al. 2011; Poulsen et al. 2011; Seeb et al. 2011). Although molecular sampling techniques have been making great advances in reducing impact on voucher specimens in the recent past (Nielsen and Hansen 2008; Gibbon et al. 2009), the same cannot be said for the majority of morphological taxonomists that still preserve their samples in formalin; an outdated practice that renders genetic analysis very difficult if not impossible (Ward et al. 2009). Many BFT larval studies have used formalin as a preservative, which severely limits future genetic verification of samples (Piccinetti et al. 1997; García et al. 2002; Koutrakis et al. 2004; Alemany et al. 2006; Isari et al. 2008; Alemany et al. 2010; Catalán et al. 2011; Koched et al. 2013). After discovering the way in which formalin corrupts the body shape and pigmentation of larvae, Ueyanagi (1966; 1969) began preserving their larvae in 70% ethanol. Researchers with the California Cooperative Oceanic Fisheries Investigations use a conversion factor of 1.098 to convert from formalin fixed length to live length because of formalin-induced shrinkage of larvae (Lo et al. 2010). Admittedly, ethanol also distorts diagnostic morphological features; however, because it is non-toxic ethanol is less hazardous for field surveys. Therefore, we strongly suggest that future surveys store all collected samples in >70% ethanol at -20°C. Ethanol that is either denatured or purified to concentrations >96% should be avoided, due to the presence of additional chemicals that hinder DNA preservation and interfere with downstream genetic applications. Alternatively, replicate samples collected during survey tows by means of a bongo net, or Folsom plankton splitter post-collection, can be preserved in both ethanol and formalin. Researchers working with nematodes have found that DESS, a solution containing a few common laboratory chemicals, is capable of preserving morphological features and genetic integrity for extended periods of time at room temperature (Yoder et al. 2006).

Finally, we suggest that all routine fisheries work involving larvae should make use of taxonomists and geneticists in order to ensure both accuracy of results and efficient use of financial resources. Genetic barcoding and NGS techniques are legitimate tools that can support species identification and play a crucial role in fisheries management efforts.

Acknowledgements

This work was co-funded by the MARES Joint Doctorate programme selected under Erasmus Mundus and coordinated by Ghent University (FPA 2011-0016). Many thanks to Antonio Di Natale, Jerry Scott, John Lamkin and Edorta Aranguena for helping to assemble the various tuna taxonomic keys. Thanks are also due to the IEO researchers in

charge of the tuna rearing experiments at the IEO facilities in Mazarrón, F. de la Gándara, A. Ortega and P. Reglero, for providing the images of BFT larvae.

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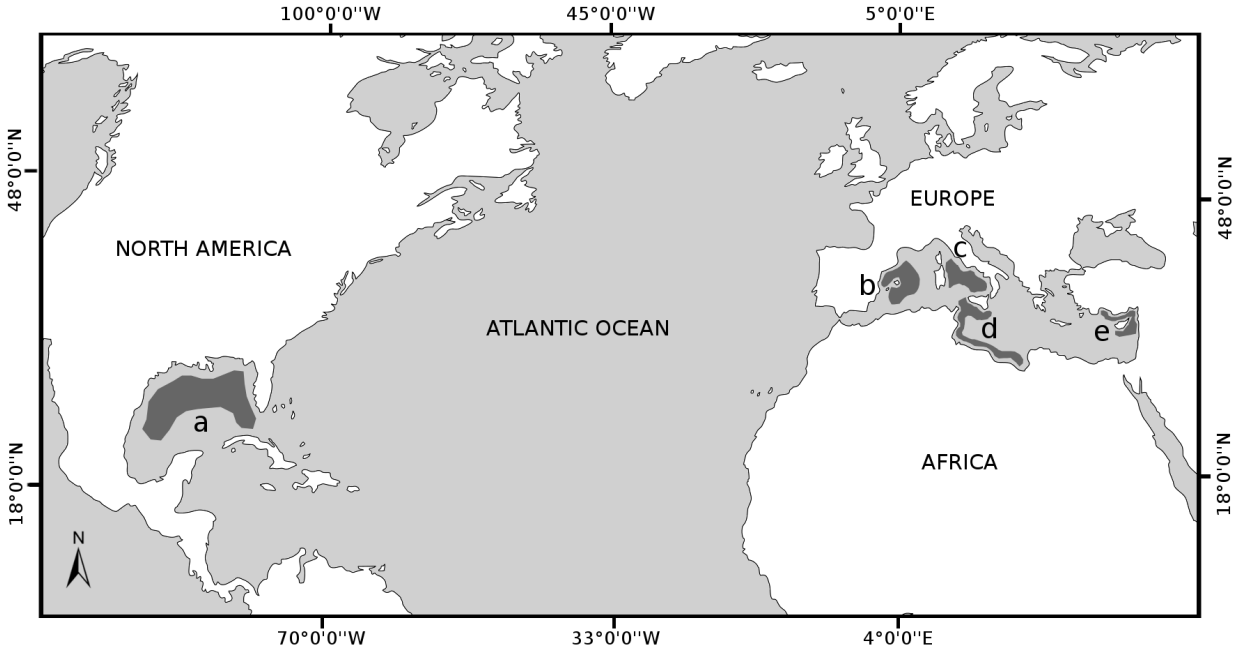

Fig. 1 Map showing known spawning areas (shaded polygons) of the Atlantic bluefin tuna in the Gulf of Mexico (a), Balearic Sea (b), Tyrrhenian Sea (c), Ionian Sea (d) and Levantine Sea (e).

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Fig. 2 Photographs of Altlantic bluefin tuna (*Thunnus thynnus*) larvae reared from eggs collected from spawning induction cages at El Gorguel (Cartagena, Spain) and cultivated at the larval rearing plant at the Spanish Institute of Oceanography in Mazarrón (de la Gándara et al. 2010). Larval developmental stages represented are: yolk sac (a), 8 days post hatching (b), and 14 days post hatching (c). Photographs taken by F. de la Gándara and adapted for publication using GNU Image Manipulation Program 2.8.14.

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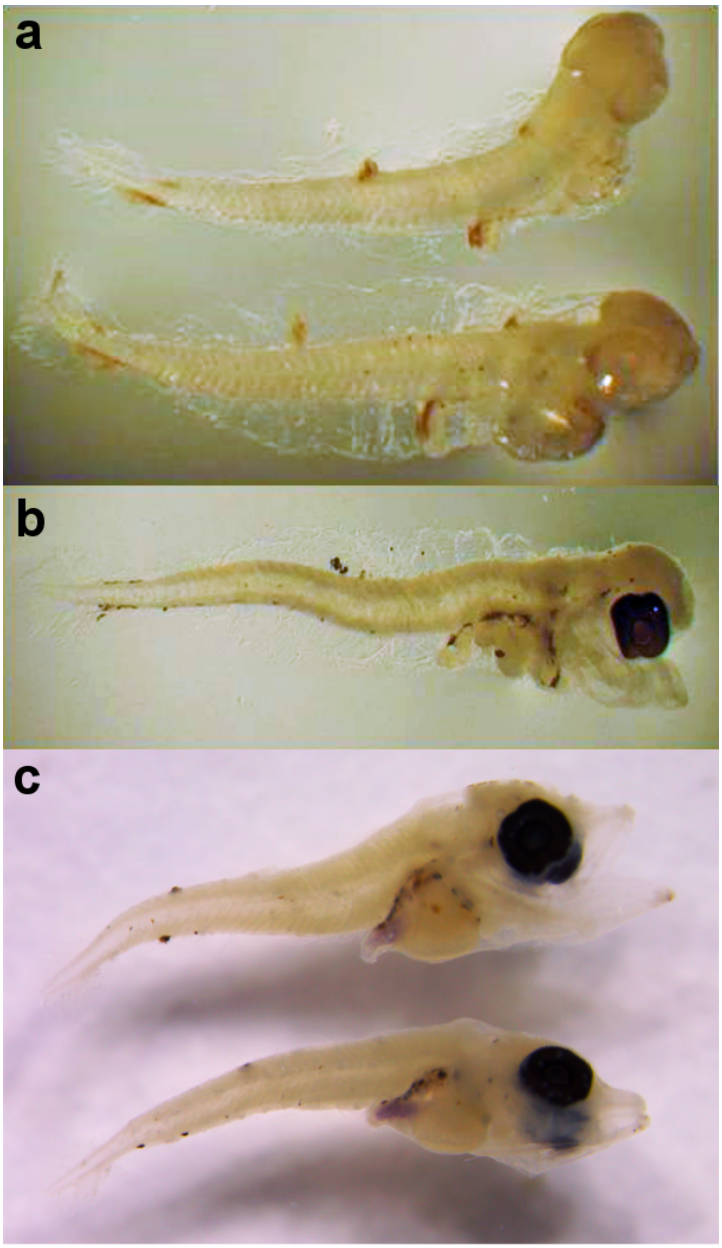

Fig. 3 Photographs of Altlantic bluefin tuna (*Thunnus thynnus*) larvae captured in the Balearic Sea and preserved in formalin. Approximate age of larvae are: <24h post hatching yolk sac larvae (a), 24h post hatching (b), 48h post hatching (c). All photographs were taken by F. Alemany and adapted for publication using GNU Image Manipulation Program 2.8.14.